

Neuronal Reprogramming via Nanochannel Electroporation

Honors Research Thesis

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Abstract

A literature review of neural development and cell reprogramming gives insight into the complexity of the mechanisms that take place during differentiation. Many technologies for transfection exist, however each has its own drawbacks. Most commonly, low efficiency rates, high cytotoxicity, expensive costs, transient transfections, and high variability are disadvantages to traditional methods of transfection. The novel technology of nanochannel electroporation (NEP) solves a wide range of these issues with significantly higher efficiency rates and low cell-to-cell variability through single cell transfection. NEP done on mouse embryonic fibroblasts (MEFs) with transcription factors *Brn2*, *Ascl1*, and *Myt1l* (BAM) found that addition of particular patterning genes to BAM-mediated reprogramming increased induced neuron efficiency and complexity. Additionally a possible dependence on the cell cycle and a dedifferentiation to a progenitor-like stage were demonstrated during neuronal reprogramming. The results reveal a stochastic nature to BAM-mediated neuronal reprogramming, allowing for a greater understanding of cell reprogramming and its applications in regenerative medicine.

Keywords: differentiation, transfection, regenerative medicine, induced neurons

Chapter 1: Introduction and Literature Review

Embryonic and Neural Development

During the development of higher animals, the blastula is reorganized into a gastrula in a process called gastrulation. Throughout gastrulation, the embryonic stem cells differentiate into three layers of cells including the endoderm, the mesoderm, and the ectoderm. Each germ layer then differentiates further into specific tissues and cells. For example, the cells in the ectoderm develop into the epidermis and neural cells, those in the mesoderm develop into muscles and the cardiovascular system, and those in the endoderm become the gut and the liver (Kimelman, 2011). These developmental processes are coordinated by specific gene expression patterns, dictated by the nature of the DNA itself as well as by proteins that enhance or repress transcription.

Morphogens are signaling molecules secreted by cells that influence gene expression and differentiation in embryonic development (Wolpert, 1996). According to the gradient morphogen hypothesis, morphogens act as positional cues that form a signaling gradient to regulate differential gene expression in a concentration dependent manner (Ashe & Briscoe, 2006). Thus the morphogens set up concentration gradients that drive the specialization of stem cells into tissues. The mechanism behind the gradient morphogen hypothesis has still not been determined, but some possibilities are the use of binding site affinity, combinatorial inputs, feed-forward loop, or positive feedback.

The gradient morphogen hypothesis plays a large role in neural development. In vertebrates, the hindbrain drives essential basic functions including motor activity, blood circulation, and rhythmic breathing (Moens & Prince, 2002). The development and organization of the hindbrain is highly conserved evolutionarily, segmented into seven rostro-caudal (RC) regions with specific functional roles. These unique segments termed rhombomeres, result from

specific patterns of cell proliferation mediated by morphogens. Each rhombomere has corresponding progenitor cells with patterning genes expressed in similar patterns as the original rhombomeres. In addition to RC patterning, there is also dorso-ventral (DV) patterning. Together the RC-DV patterning factors form axes specifying the location of all cells in the hindbrain.

Cell Reprogramming

The previous viewpoint supposed once cells had undergone differentiation, they were permanently altered from stem cells. However in the 1960's, scientists established somatic cell nuclear transfer (SCNT), injection of a nucleus from a fully differentiated adult cell into an enucleated egg resulting in an embryo. Thus scientists comprehended that the nucleus contained all the necessary genetic information to form an entire organism and the possibilities of cell reprogramming began. In 2006, a landmark study showed that adult mouse fibroblasts could be dedifferentiated into induced pluripotent stem (iPS) cells (Takahashi & Yamanaka, 2006). This method was strikingly simple, with expression of only four exogenous transcription factors needed. Although a significant breakthrough for basic science, cell-based therapies using iPS cells would likely require differentiation of iPS cells into the lineage of interest. For this reason, many researchers looked into methods to reprogram cells directly without the iPS cell intermediate.

Then in 2010, an article demonstrated that three RC-DV transcription factors converted adult mouse fibroblasts into induced neuronal cells (Vierbuchen et al., 2010). This differed from other cell reprogramming studies, since fibroblasts and neurons are from separate cell lineages, mesoderm and ectoderm, respectively. The three exogenous transcription factors used were *Brn2*, *Ascl1*, and *Myt1l* (BAM), transforming genes involved in neuronal development or function (Figure 1). Additionally, *Ascl1* alone was found to reprogram cells with immature neuronal

features (Vierbuchen et al., 2010). Additionally, the specific fates of neurons may be able to be induced by including additional RC-DV patterning genes through transfection (Hirai et al., 2011)

Transfection Technologies

Transfection is the artificial introduction and uptake of exogenous nucleic acids into cells (Wang et al., 2010). Transfection can be either transient or stable; in transient transfection the nucleic acid only remains present in the cell for a short period of time, never integrating into the genome. Transient nucleic acids can be lost after cell divisions or be degraded and do not pass from generation to generation. On the other hand, stable transfection allows the nucleic acid, usually DNA, to be incorporated into the genome of the cell. When compared to circular DNA, linear DNA is more difficult to uptake into cells, but more easily incorporated into the genome. With this method, hereditary transfected DNA can be measured by selective markers, such as antibiotic resistance. There are many chemical, lipid, physical, and viral technologies for transfection, each having its own benefits and drawbacks.

Chemical methods of transfection have been studied for many years. One of the first methods, diethylaminoethyl (DEAE)-dextran, a cationic polymer, provides use for transfection of nucleic acids into cultured mammalian cells (Vaheri & Pagano, 1965). DEAE-dextran tightly binds to negatively charged nucleic acids, resulting in an overall positive complex, allowing the complex to associate with the plasma membrane of the cell and be taken up by endocytosis. Although transfection by DEAE-dextran is relatively simple and low cost, it only results in transient transfection, not stable transfection, with high cytotoxicity and low transfection efficiency.

Another chemical method of transfection is calcium phosphate co-precipitation. During this process a mixture of the nucleic acid, frequently DNA, and calcium chloride is added to a buffered saline-phosphate solution. When the produced precipitate is added to cultured cells, they

uptake it with endocytosis or phagocytosis (Graham & van der Eb, 1973). This method can be used for both transient and stable transfection. However, a major shortcoming of using calcium phosphate co-precipitation results from the high sensitivity to slight alterations in pH, temperature, and buffer salt concentration. Additionally, calcium phosphate co-precipitation cannot be used for *in vivo* gene transfer.

Lipids can also be used for transfection; liposomes are lipid bilayers that naturally form micelles in an aqueous medium. Artificial liposomes aid in transfection, made to envelop nucleic acids, then fuse with the plasma membrane to deliver the load (Fraley et al., 1980). The next advancement has been using artificial cationic lipids. The positively charged head of the lipid interacts with the negatively charged phosphate backbone of nucleic acids to form a condensed nucleic acid-cationic lipid reagent complex. This highly efficient manner of gene transfer uses endocytosis for the uptake of nucleic acids. Some advantages are the ability to transfect a wide variety of cell lines, a broad size of nucleic acids, and transient and stable transfections. Cationic lipid transfection also functions for *in vivo* applications. However, the main disadvantage is the dependency on the cell type and culture conditions, requiring the optimization for each cell type and transfection reagent.

Physical methods of transfection include direct microinjection into cultured cells. Microinjection utilizes a thin needle, an effective but extremely time-consuming and laborious technique of gene transfer (Cappechi, 1980). Another physical method of transfection is electroporation, also called electropermeabilization (Wong & Neumann, 1982). During electroporation, a pulse of high voltage applied to cells for a short period of time increases transmembrane potential. Consequently formed nanopores of varying sizes allow nucleic acids to enter the cell. The nanopores may be temporary or irreversible, depending on the electrical

conditions and the cell type (Shigekawa & Dower, 1988) Electroporation was traditionally bulk electroporation (BEP), where a homogenous electric field applied to a solution containing cells and nucleic acids induced nanopores. For electroporation, optimal conditions for transfection must first be determined, including voltage, time of pulse, and concentrations in the solution. However, once the conditions have been established, electroporation is simple, cost-effective, fast, and can transfect a large number of cells at one time. Drawbacks of electroporation include the large amount of cell death that occurs and the damage to the cell membrane. A novel combination of various techniques is called nucleofection, an electroporation-based transfection method that enables the DNA to directly enter the nucleus (Gresch et al., 2004).

For cell types that do not uptake nucleic acids by the previously mentioned methods, another gene delivery technique is the use of viruses. This alternate method often uses adenoviral and retroviral vectors to infect cells with nucleic acids. Viral gene delivery can be used on cultured cells and *in vivo*, can result in stable transfection, and have high efficiency rates. (Vorburger & Hunt, 2002). However, some drawbacks of viral gene delivery techniques are the high costs, complex protocols, cytotoxicity, and safety concerns. Another novel method of transfection, the microfluidic platform, does not rely on viral vectors or electric fields. With this approach, cells undergo a large amount of shear stress and compression as they pass through a constriction device resulting in transient nanopores forming. Then molecules in the surrounding buffer can enter the cell (Shin, 2009). This method is being further investigated to be able to understand and utilize it.

Epigenetics

Gene expression during differentiation is not only dependent on the string of nucleotides that constitute the genomic DNA sequence. The field of epigenetics studies mechanisms that alter gene expression without changing the DNA sequence. Epigenetics are the mechanisms that alter

gene expression without changing the DNA sequence (Bird, 2007). However, epigenetic changes can be heritable between cells and sometimes from parent to offspring, essential for many cell functions, including X chromosome inactivation in some animals, but especially organismal development and cell differentiation (Eccleston et al., 2007). Epigenetics involves both intracellular and environmental influences on gene expression. Some examples of epigenetic changes are DNA methylation, histone modifications, nucleosome positioning, non-coding RNA-mediated gene-silencing, and ATP-dependent chromatin remodeling (Baylin & Schuebel, 2007).

During DNA methylation, a methyl group is added to a cytosine, creating 5-methylcytosine (Auclair & Weber, 2012). The enzyme DNA methyltransferase catalyzes this reaction, most commonly occurring at CG dinucleotides. Cytosine methylation mainly occurs as a DNA transcription inhibitor. Preventing binding of transcription factors and recruiting methylbinding proteins (MBPs), which change the chromatin environment, repress transcriptional initiation. Specific patterns in methylation are required for normal development and cell differentiation; many diseases have alterations in cytosine methylation. For example, many cancers have hypomethylation occurring in oncogenes and hypermethylation occurring in tumor suppressors (Esteller, 2007).

Another method of gene regulation is by modifying histones. Histones are alkaline proteins used for packaging DNA into a smaller structure. Negatively charged DNA wraps around the histone to form nucleosomes. These nucleosomes, connected by linker DNA, wind together to form condensed chromatin (Figure 2). Histones modification also occurs through methylation, acylation, deacetylation, and more (Schones & Zhao, 2008). Acylation consists of adding an acyl group ($-\text{COCH}_3$) to a lysine. This adds a negative charge to the positive amino acid, thus reducing the attraction between the histone and the DNA sequence. Therefore, acetylation results in a less

tightly packed nucleosome and increases ability for active transcription (Vidali, Gershey, & Allfrey, 1968). On the other hand, deacylation results in more intermolecular forces between the negative DNA sequence and the positive lysine of the histone, resulting in a tighter structure.

The modifications of certain nucleotides and amino acids are not the only epigenetic changes that affect gene expression. The highly complex structure of the chromatin allows for DNA to be packaged into a cell, prevents DNA damage, aids in DNA replication, and results in higher or lower rates of transcription in certain areas. Chromatin tightly bound in complex forms is referred to as heterochromatin. Therefore, the DNA sequence becomes less accessible, and the binding proteins and transcription factors are less likely to bind to the sequence for initiating transcription (Schones & Zhao, 2008). Thus the DNA in heterochromatin is generally inactive with reduced gene expression. On the other hand, less densely packed euchromatin has more accessible genes being actively transcribed. Euchromatin contains the majority of coding genes in the genome. Heterochromatin and euchromatin can be differentiated with staining, heterochromatin stains more intensely and darker and has been found to be at the extremes of the nucleus and chromosomes (Mello, 1983). Evidently, there are many mechanisms of epigenetic modifications including cytosine methylation, histone acylation, chromatin structure modifications, and more, that need to be taken into account when doing molecular biology.

Chapter 2: Methodology

Nanochannel Electroporation

In the literature review, various methods of transfection were discussed, all having some advantages and some disadvantages. A novel technology for transfection has been created using electroporation of cultured cells through a 3D array of nanochannels in a modified Transwell insert (Figure 4). Nanochannel electroporation (NEP) allows for the transfection of many single cells simultaneously by applying a high electric field to a small portion of the cell membrane (Boukany et al., 2011). NEP conditions can be adjusted to control the amount of molecules delivered at the single cell level, with high transfection efficiencies and low cell-to-cell variability. Therefore, NEP is an optimal gene delivery technology capable of introducing complex combinations of DNA into a large number of individual cells without the need for a needle.

Plasmid Preparation

The unique features of the NEP technology allow us to reprogram cells by delivering DNA expression plasmids into cells. The plasmids need to be equipped with hindbrain genes as depicted by the RC-DV patterning. The strategy began with cloning the transcription factors into plasmids of DNA containing mammalian promoters and antibiotic resistance. Restriction enzymes proteins cleave DNA at specific nucleotide sequences and DNA ligase connects two strands of DNA together by forming new phosphodiester bonds (Roberts, 1976). Thus, to clone the transcription factors into the vectors, restriction enzymes cleaved both the vector and the transcription factor and ligated them together. The final plasmid constructs then had to be amplified and purified. A standard transformation protocol transferred the plasmids into NEB 5-alpha competent *E. coli* cells. The cells proliferated on ampicillin media, since the cells that received the plasmid successfully acquired antibiotic resistance. Single colonies were harvested and allowed to grow in 250 mL of

SOC (super optimal broth) media with ampicillin. A maxi-prep isolated the DNA plasmids for each transcription factor to, and then lastly each sample was purified. Then Daniel Gallego-Perez at The Ohio State University College of Engineering used the plasmids for BAM-mediated cell reprogramming of MEFs (mouse embryonic fibroblasts) into neurons via NEP.

Immunohistochemistry

Antibodies, or immunoglobulin molecules, are proteins created by the immune system for binding to a specific portion of a protein, called an antigen. Generally, antibodies recognize foreign substances and tag them for degradation (Buchwallow & Bocker, 2010). However, antibodies can also be generated for antigens within the body, or self-antigens. Immunohistochemistry (IHC) utilizes antibodies to detect antigens and specific proteins in biological tissues (Coons et al., 1941). The procedure used for IHC begins with preparation of the tissue. MEFs were isolated from embryos around the developmental stages of E12 were plated directly on the Transwell for NEP.

After NEP, once the cells were ready for IHC staining, the tissue incubated in 0.1% 100x Triton in PBS 1x buffer for 5 minutes for permeabilization. Meanwhile a blocking solution was made, containing 0.1% 100x Triton and 5.0% goat serum in PBS 1x buffer. The goat serum acts as a blocking agent for nonspecific sites. The tissues incubated in the blocking solution for half an hour, while preparing the primary antibody solution. The primary antibody binds directly to the antigens in the tissue sections (Hewitt et al., 2011), with a dilution between 1:1000 and 1:200 in blocking solution. After overnight incubation, the primary antibody solution was rinsed off, and the cells incubated in a 1:1000 dilution of the secondary antibodies and DAPI (4',6-diamidino-2-phenylindole) for one hour. The DAPI stains nuclei blue, while the secondary antibodies bind to the corresponding primary antibodies to amplify the signal (Figure 5), (Odell & Cook, 2013). The

secondary antibody can be either green (488), red (594), or far red (647); therefore, up to three proteins could be stained for at one time.

Confocal Fluorescence Microscopy

Confocal fluorescence microscopy techniques were used to visualize the experimental results. Fluorescent microscopes in particular utilize the fluorescence given off by certain compounds when they absorb light or another form of electromagnetic radiation and emit another wavelength of light (Harlow & Lane, 1999). During fluorescence, a molecule absorbs a high energy light and then emits a photon with less energy, since some energy is lost internally. Molecules that fluoresce, or fluorophores, emit light of a specific wavelength or color. Immunofluorescence staining (IF) utilizes antibodies labelled with fluorophores to be able to visualize the proteins (Odell & Cook, 2013). Then fluorescent microscopes shine on the stained tissue samples with light of a particular wavelength, and the emission of the fluorophores on the labelled secondary antibodies can be recorded. Confocal microscopes are an imaging technique allowing the proteins to be visualized at a higher optical resolution. In a confocal microscope, light reflects off mirrors and passed through a pinhole at the confocal plane of a lens. The pinhole eliminates out-of-focus light and visualizes one point at a time (Minsky, 1988). Thus scanning mirrors allow for a 3-dimensional (3D) image to be viewed on an attached computer. Therefore, using a confocal fluorescent microscope allows for high resolution images of fluorescent secondary antibodies.

Chapter 3: Results

Addition of *PHOX2B*, *PHOX2A*, and *RUNX3* to BAM-Mediated Neuronal Reprogramming Increases Induced Neuron Efficiency and Complexity

Additional DV-RC patterning genes added to nuclear reprogramming transcription factors may induce a specific type of neuron (Hirai et al., 2011). In particular, transcription factors *PHOX2B*, *RUNX3*, and *PHOX2A* (PRP) are all involved in the DV-RC patterning of autonomic hindbrain neurons (Levanon et al., 2001, Brunet & Pattyn, 2002). Therefore, we added PRP plasmids with BAM to the NEP cocktail for E12.5 MEFs and measured the relative efficiency of neuronal reprogramming by staining for TUJ1, a neuronal marker. The efficiency of BAM-mediated NEP with added PRP was significantly higher than the control condition (Figure 6A,B,C). However, even more notable was the difference in neuronal complexity of the cells in the BAM-PRP condition. Using Neurolucida computer software for a Sholl analysis and calculating the dendrite complexity index verified that the induced neurons with the PRP added to the NEP cocktail were significantly more complex (Figure 6D,E). Therefore, it can be concluded that neuronal reprogramming efficiency and complexity can be significantly increased by adding additional DV-RC transcription factors to the NEP cocktail.

Early *CCNA2* Deletion Decreases Induced Neurons

Cyclins regulate the cell cycle by activating cyclin-dependent kinases. Cyclin A2, from the gene *CCNA2*, promotes DNA replication, particularly for the onset of the S-phase (Girard et al., 1991). Research has shown that *CCNA2* function is essential for cell cycle progression of hematopoietic and embryonic stem cells (Kalaszczynska et al., 2009), yet the role of the cell cycle in neuronal reprogramming is unknown. Deletion of the gene *CCNA2* will aid in determining the role of cyclin A2 in BAM-mediated neuronal reprogramming. To ablate *CCNA2*, we acquired

CCNA2^{fl/fl} MEFs from pups with the *CCNA2* allele flanked by lox (floxed), which results in removal of the gene after *cre*-mediated recombination. Therefore the MEFs underwent NEP with BAM and *cre*-GFP and 14 days later were assayed for neuronal reprogramming by staining for TUJ1. Wild type MEFs underwent reprogramming, by staining positive for TUJ1, but *CCNA2*^{fl/fl} MEFs expressing *cre*-recombinase were negative for TUJ1 (Figure 7D,E). In separate experiments, MEFs were infected with adenovirus *cre*-GFP 6 hours after the onset of the NEP, so that the MEFs would have at least 6 hours of time from exposure to BAM mRNA to *cre*-mediated recombination. BAM-treated MEFs infected with adenovirus 6 hours post-NEP stained positive for TUJ1 (Figure 7F)). We conclude that early deletion of *CCNA2* reduced BAM-mediated nuclear reprogramming, so onset of S-phase or other *CCNA2* functions are required for neuronal reprogramming.

A Subset of Induced Neurons Undergo a Nestin-Positive Stage

Since cyclin A2, essential in S-phase, was found to be necessary for BAM-mediated neuronal reprogramming, the cells may be undergoing S-phase during the reprogramming. However, mature neurons rarely undergo S-phase, so it is possible that the somatic fibroblasts first undergo dedifferentiation and then pass through a neural stem cell phase. Then this neuronal progenitor phase may be followed sequentially by differentiation to the induced neurons. If this occurs, then there is a stochastic nature to the differentiation of cells, since progenitor cells can also differentiate to astrocytes. Furthermore, such a finding would indicate that BAM-mediated induced neuron formation does not represent direct conversion from fibroblasts into neurons. With this in mind, we stained MEFs that had undergone BAM-mediated NEP and found that many cells stained positive for GFAP, a marker for astrocytes, but not for GFP, a marker for *Ascl1*. The GFP-positive/GFAP-negative cells showed morphology consistent with neurons,

indicating that *Ascl1* expressing cells at this stage had not differentiated into astrocytes (Figure 8A,B). To test the possibility that the MEFs undergo a neural progenitor cell stage, we stained cells after NEP for nestin, an intermediate filament expressed in neural stem cells, and found many cells stained positive for nestin and GFP (Figure 8C). Then we evaluated mRNA expression of nestin in cells that had undergone BAM-mediated neuronal reprogramming by quantitative rt-PCR. There was a significant increase in nestin expression 24h post-NEP, indicating that BAM transfection directly or indirectly induces nestin expression. Additionally, *Ascl1* expression positively correlated with nestin expression and increasing NEP pulse number increased nestin expression (Figure 8D). Next MEFs were made with two specific loci, *nestin-cre-ER* and *Rosa^{mTdTomato/mGFP}*. Alone these MEFs will express TdTomato, but in the presence of *cre*, deletion of TdTomato and expression of GFP occurs. *Cre* recombinase will only function in nestin-positive cells that have been treated with 1 μ M 4-OH (tamoxifen), due to the fusion of *cre* to an estrogen receptor (ER). This experiment resulted in a significant increase of cells staining positive for GFP (Figure 8E,F,G,H), demonstrating that a subset of cells pass through a nestin-positive stage. Furthermore, we identified several TUJ1-positive neurons that were also GFP positive, indicating that these neurons had passed through a nestin-positive stage.

Chapter 4: Discussion

Stochastic Barriers of BAM-Mediated Neuronal Reprogramming

Various implications arise from the results presented in this project; firstly we demonstrated that adding plasmids of *PHOX2B*, *RUNX3*, and *PHOX2A* to BAM increased the neuronal reprogramming efficiency significantly. However, the increase in induced neurons was relatively small. Therefore, we conclude that there are other factors that regulate induced neuron efficiency downstream of exogenous plasmid delivery. Even then, since the BAM-PRP condition contained neurons with significantly more complexity, we have shown that the dorso-ventral and rostro-caudal patterning axes are important in neuronal reprogramming. Since early deletion of *CCNA2* resulted in a decrease of induced neurons, cyclin A2 must be necessary for BAM-mediated neuronal reprogramming. However, cells do not have cyclin A2 present at all times, only before the onset of the S cycle. Therefore, perhaps only MEFs that contain cyclin A2 and are in S phase at the time of NEP can undergo directed differentiation. This adds another barrier with a random nature to neuronal reprogramming. Lastly, the results using MEFs with the loci *nestin-cre-ER* and *Rosa^{mTdTomato/mGFP}* show that some MEFs undergoing BAM-mediated NEP pass through a nestin-positive neuronal progenitor-like stage. This implies that cells first undergo dedifferentiation and then re-differentiation with different pathways they may follow, such as to neurons or astrocytes. Again, this is another stochastic barrier to neuronal reprogramming

Significance and Future Direction

This project has advanced the field of neuronal reprogramming by helping to comprehend the stochastic nature of current transfection approaches. By understanding this phenomena, measures can be taken during neuronal reprogramming to reduce the random nature of directed differentiation. One possibility for improving the reprogramming efficiency is to create a more

complex plasmid cocktail. The use of NEP has allowed for the specific experiments done, doing single cell transfections with low variability. NEP has many uses outside of neuronal reprogramming; any charged molecule utilized in reprogramming such as RNA (Warren et al., 2010), miRNA, and drugs (Guo et al., 2013) has applications with NEP. Improving the NEP platform is a future goal, to control even more precisely the DNA intake of cells. Cell reprogramming has many implications for use in regenerative medicine and clinical applications using these techniques.

Figures and Tables

Figure 1: Routes to Cell-Lineage Reprogramming (Nicholas & Kriegstein, 2010)

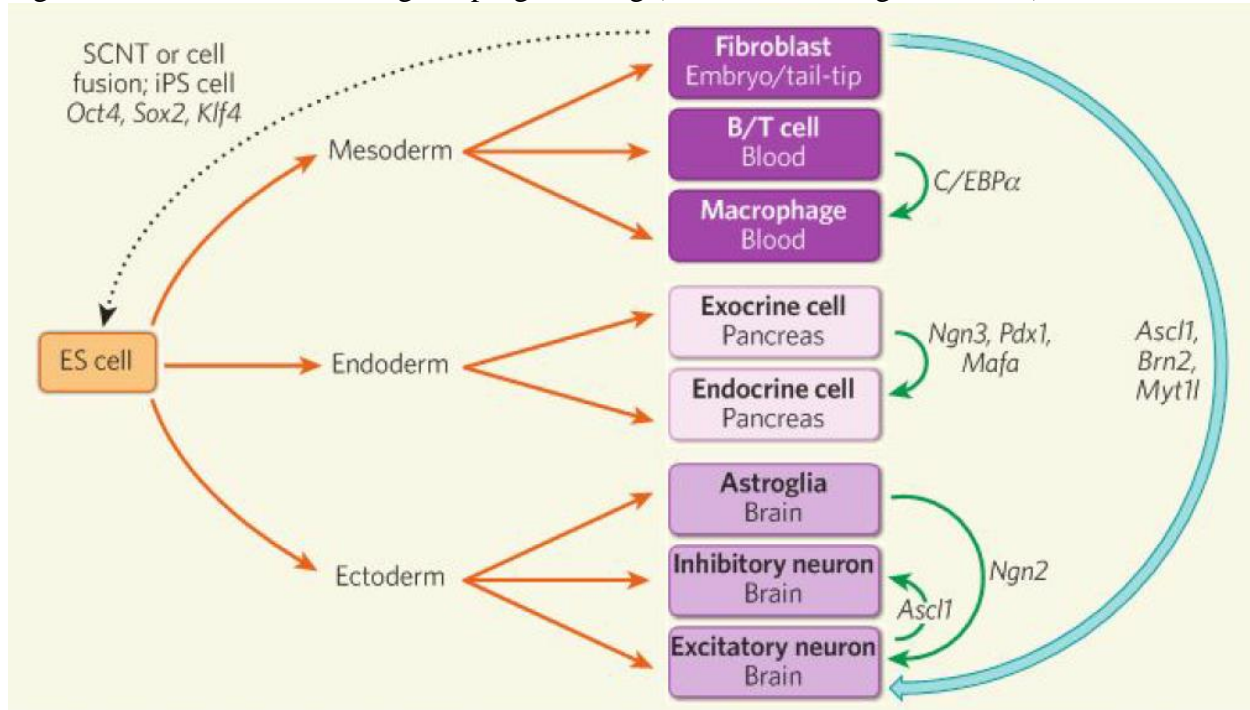


Figure 2: Genomic Architecture (Baylin & Schuebel, 2007)

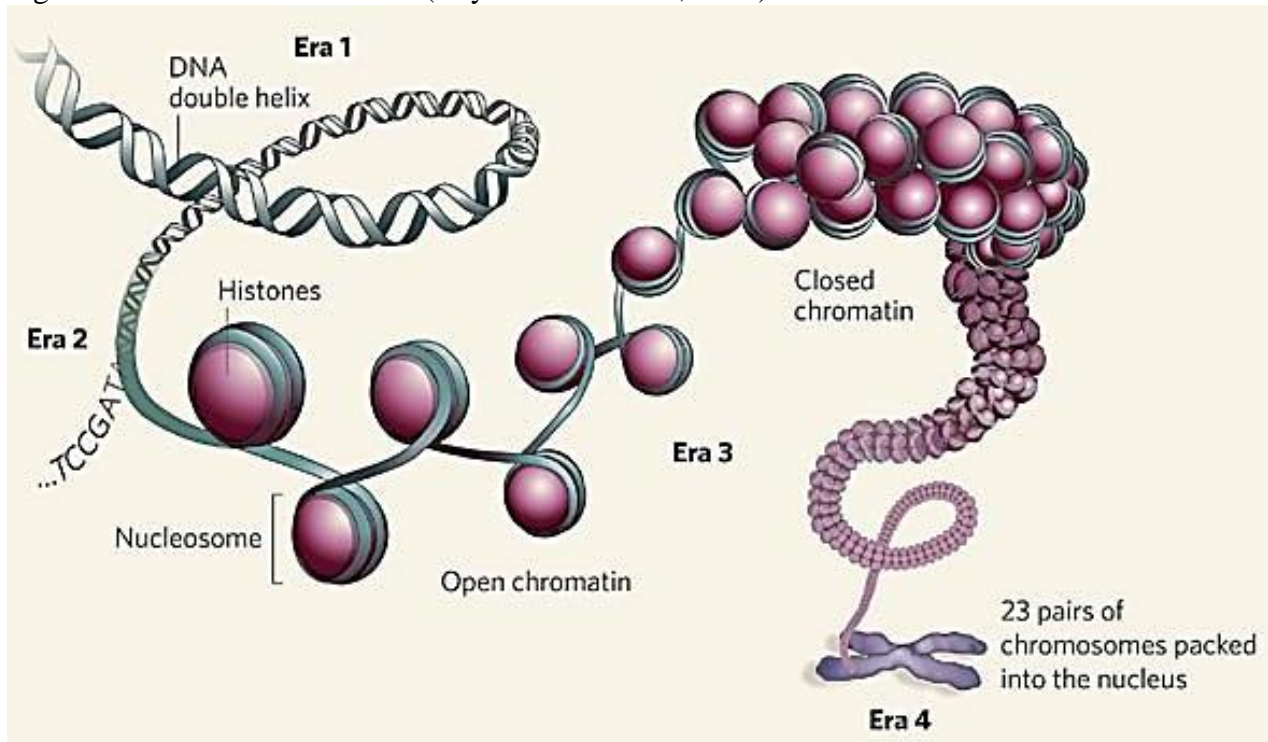


Figure 3: Molecular Pattern of Hindbrain in Embryo (Otero, 2014)

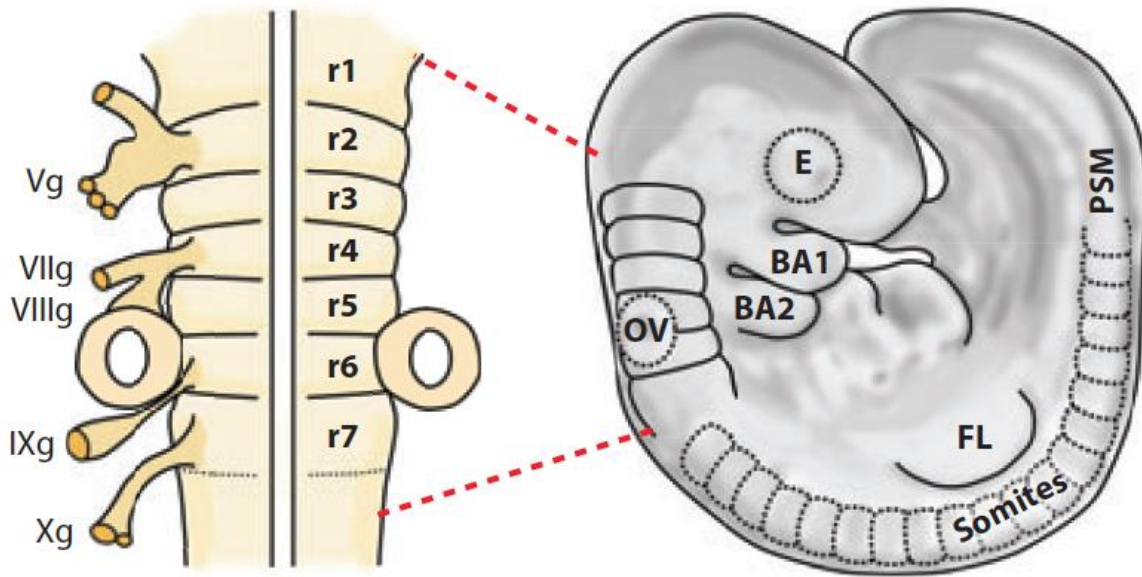


Figure 4: Transwell Modification Steps with SEM of the Microwell Array (Boukany et al., 2011)

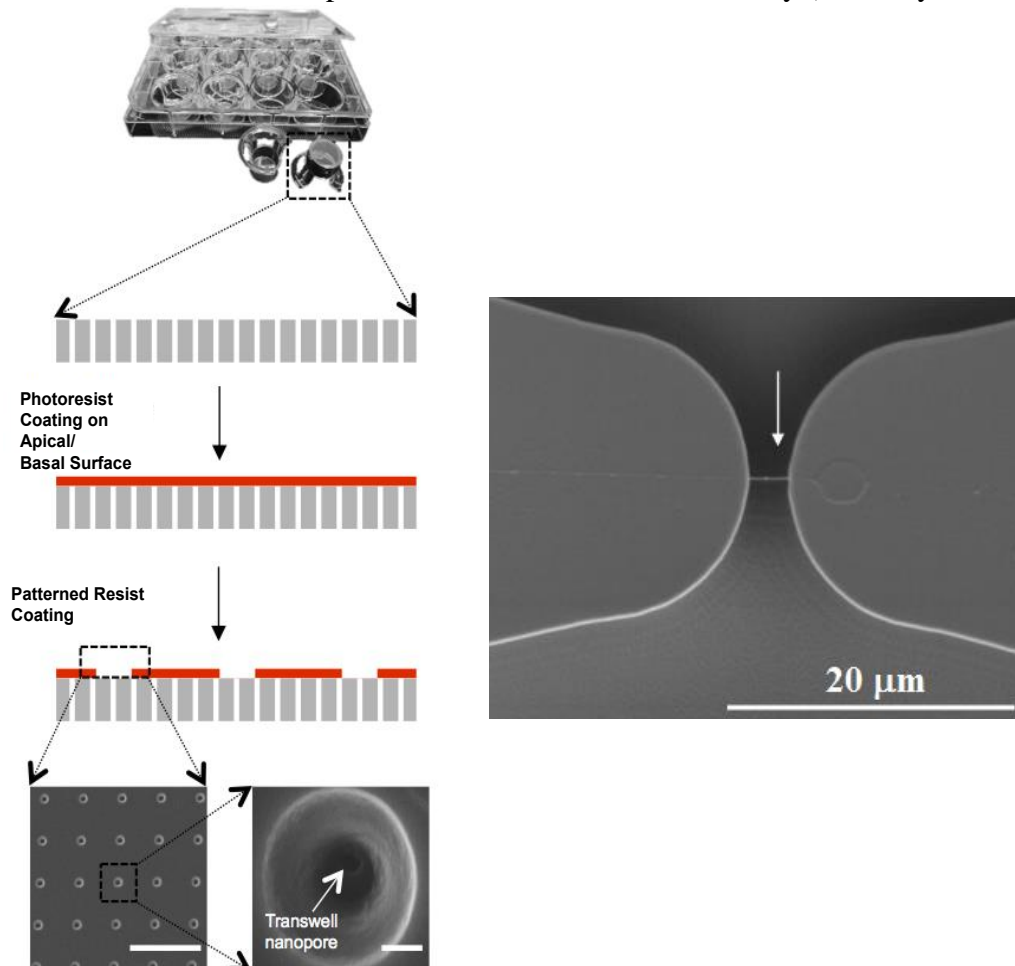


Figure 5: Antibody Interactions (Odell & Cook, 2013)

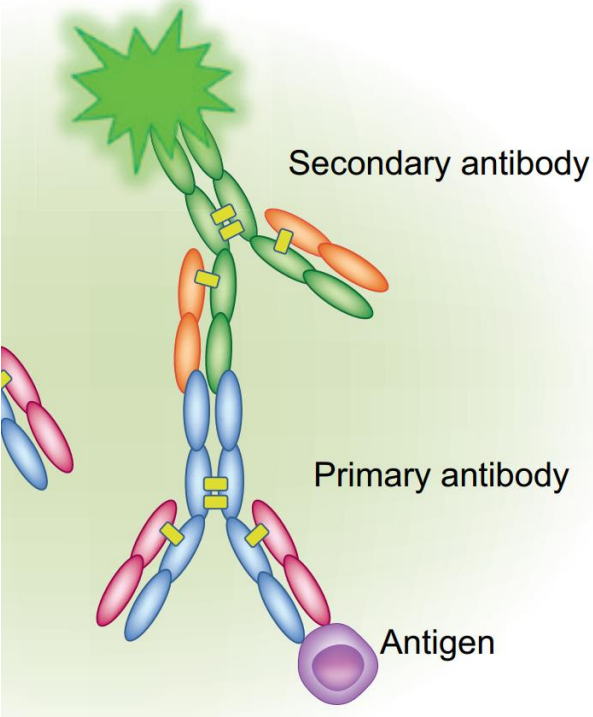


Figure 6: BAM versus BAM-PRP NEP results in increased neuronal reprogramming efficiency and complexity

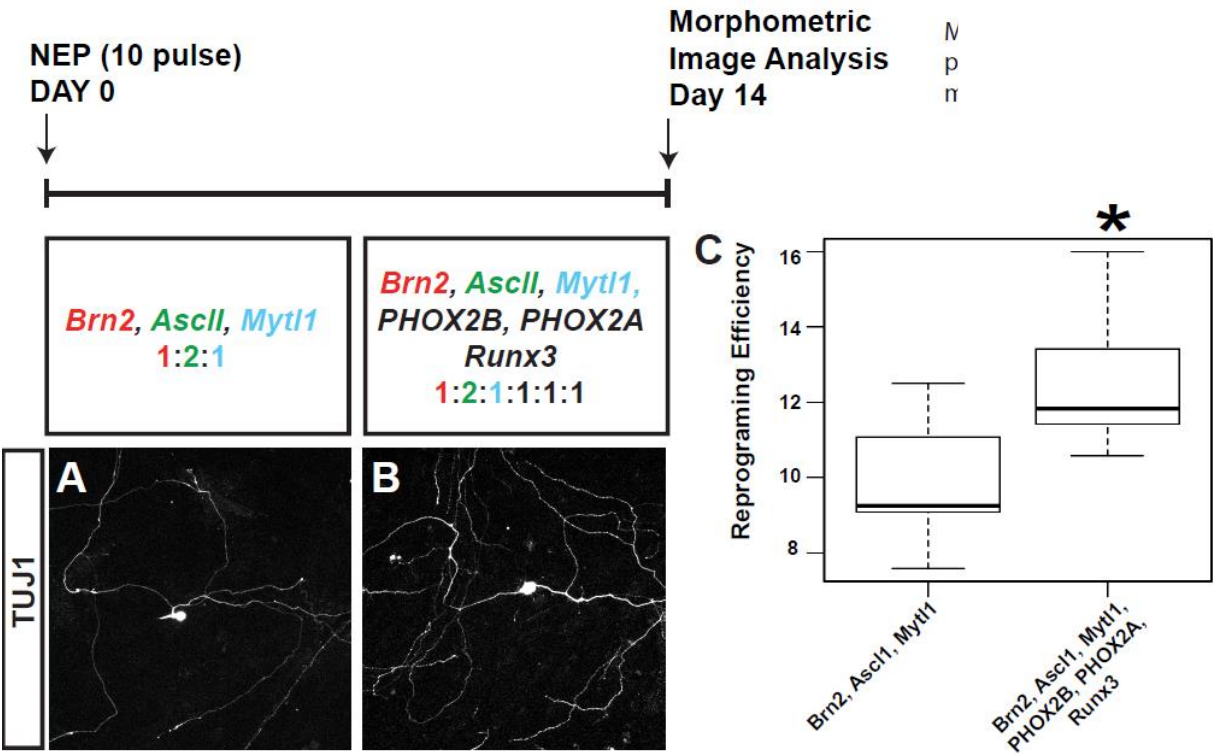


Figure 6 continued:

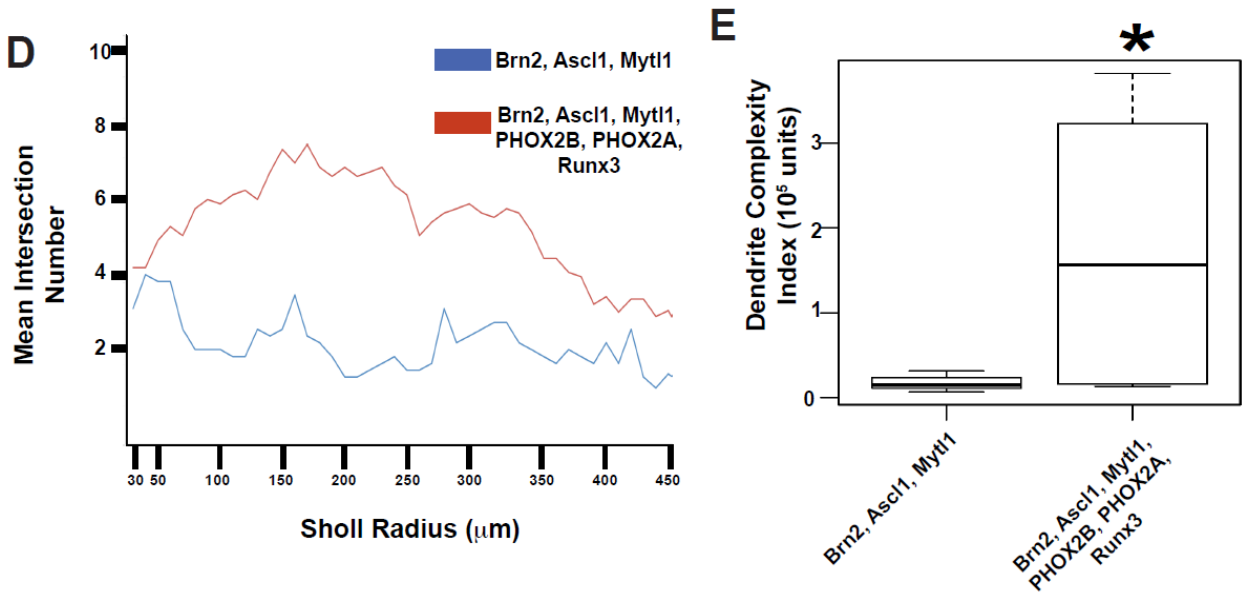


Figure 7: Early ablation of *CCNA2* decreases neuronal reprogramming

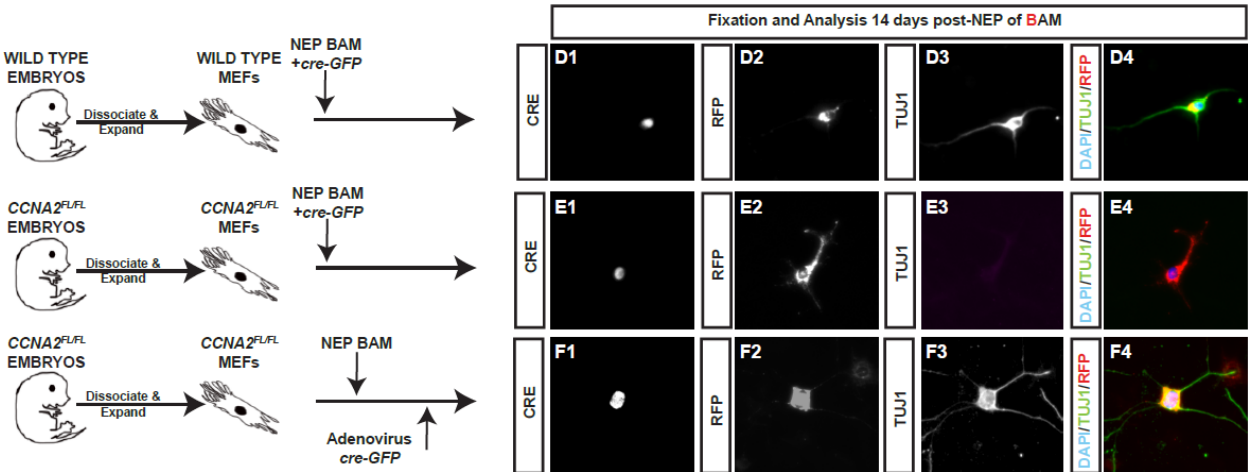
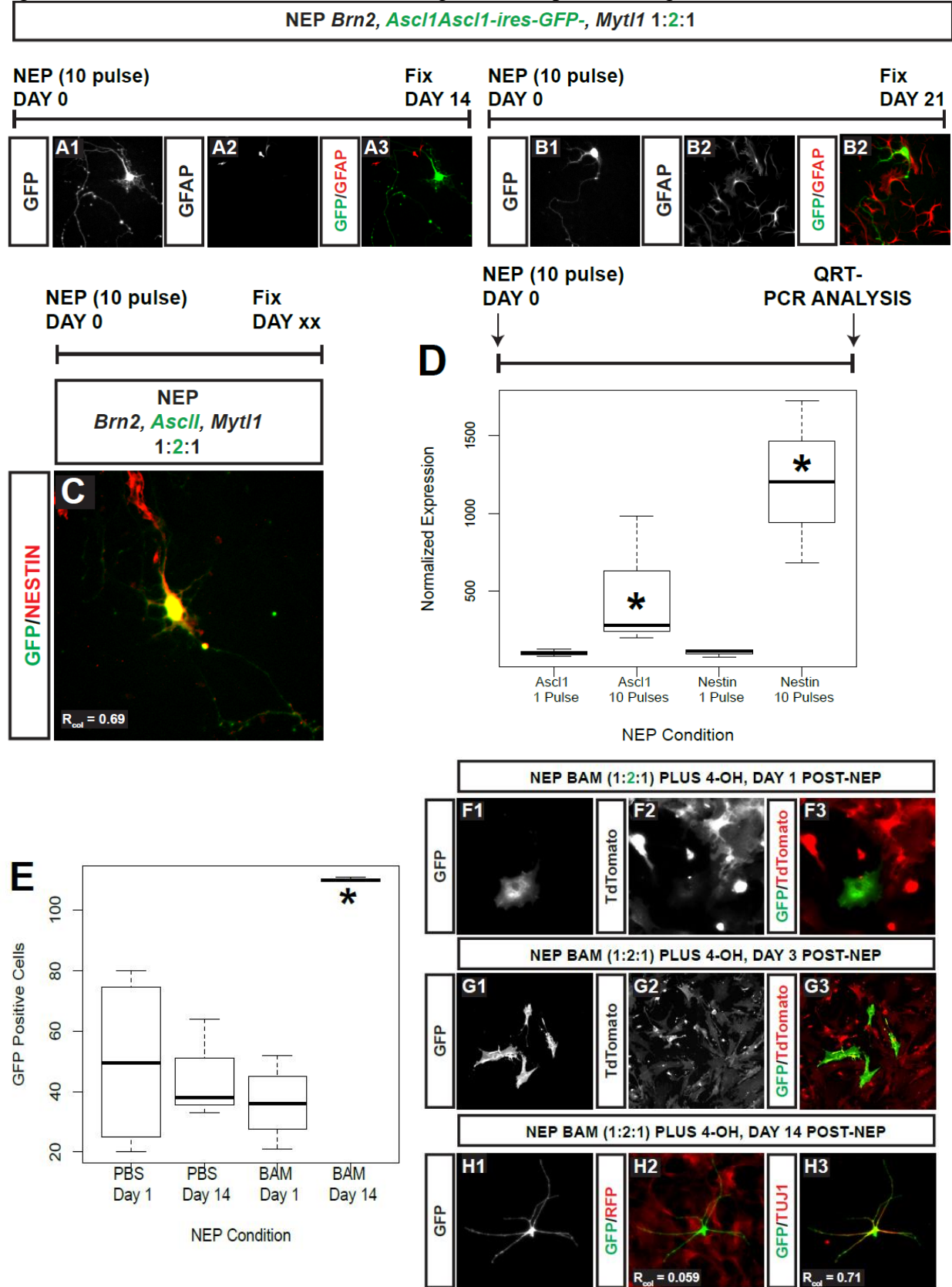


Figure 8: A subset of induced neurons undergo a nestin-positive stage



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